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Identification of a SCAR marker associated with *Bm*, the beet mosaic virus resistance gene, on chromosome 1 of sugar beet

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With 4 figures and 3 tables

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Abstract

Beet mosaic virus (BtMV) is an aphid transmitted, viral disease of beet found worldwide. The *Bm* gene, a resistance gene effective against BtMV, was identified in the sugar beet line 8500 and backcrossed into a C37 background to produce line C719. Three populations were developed from the cross of line C719 with the susceptible line C37 with the intent of developing markers for use in marker-assisted selection. The F_2 progeny of three crosses were scored for resistance. Two of the three populations conformed to a 3 : 1 ratio, indicating a single gene trait. Sequence characterized amplified region (SCAR) markers were developed by using bulked segregant analysis combined with random amplified polymorphic DNA type markers. The markers showed close association to the *Bm* resistance gene and were effective in all three populations. The A_1 allele for genetic male sterility also was found to be associated with *Bm* and the SCAR marker. Development of a single-nucleotide polymorphism marker from the SCAR sequence was used to validate linkage to chromosome 1 using separate mapping populations. This marker will be useful for the introgression of the *Bm* gene into germplasm.

Key words: *Beta vulgaris* — RAPD — SNP — virus yellows

Beet mosaic virus (BtMV) is one of the most widely distributed sugar beet viruses and is present in all major sugar-beet-producing regions of the world (Whitney and Duffus 1986, Kaffka and Lewellen 2001). In field inoculation trials, root yield losses have been documented as high as 20% (Shepherd et al. 1964, Shepherd and Till 1965). Beet mosaic virus is commonly associated with virus yellows of sugar beet and its pathogenic effects are additive to those caused by virus yellows (Shepherd et al. 1964). Moreover, in greenhouse tests of individual virus and mixed virus infections using BtMV and components of virus yellows, Wintermantel (2005) showed that, based on symptom expression, plant biomass and virus titer, the coinfection of BtMV with the yellowing viruses was synergistic. By itself, symptoms induced in sugar beet by the virus include a light green appearance to yellow mottling of the beet leaf (Shepherd and Till 1965). Recently, the RNA genome of BtMV has been completely sequenced and has been shown to be a distinct species of the genus *Potyvirus* (Nemchinov et al. 2004).

Beet mosaic virus is transmitted in the field by many species of aphids, typically the green peach aphid [*Myzus persicae* (Sulzer)], but can also be mechanically transmitted in artificial inoculation studies. Dusi and Peters (1999) showed that BtMV infected plants could be used as inoculum sources throughout the growing season and that BtMV could be vectored by at least six insect species including *Myzus persicae*. Dusi and Peters (1999) also showed that latency and incubation periods were reduced with increased temperature and leaf growth rate.

Lewellen (1973) was the first to study the inheritance of BtMV resistance in sugar beet. Inheritance studies concluded that one dominant or incompletely dominant gene, *Bm*, conditioned resistance to all the isolates of BtMV tested (Lewellen 1973). This study also showed that resistance did not condition immunity and concentrations of the virus were reduced by the *Bm* gene, with the heterozygous F_1 having a slightly higher virus concentration than the homozygous parent.

In the present work, it was observed that the *Bm* gene appeared to be associated with nuclear-encoded male sterility. Prior to the development of molecular markers, the morphological genetic factor for Mendelian male sterility (A_1) (Owen 1952) had been assigned to linkage group III of *Beta vulgaris* L. (Theurer 1968b), corresponding to chromosome 1 according to Butterfass (Schondelmaier and Jung 1997). Mendelian or genetic male sterility was the only known marker for this linkage group. Attempts to identify other morphological markers linked to A_1 have been unsuccessful (Theurer 1968a, Stander and Theurer 1970). In the present study, molecular and morphological markers linked to the *Bm* gene were identified in three populations segregating for resistance to BtMV. Sequence characterized amplified region (SCAR) markers were also developed for use in marker-assisted selection for the introgression of this gene into new sugar beet germplasm.

Materials and Methods

Plant materials: Following the original genetic analysis for resistance to BtMV, the *Bm* allele was backcrossed into the breeding line C37 (Lewellen et al. 1995). Germplasm line C719, containing a high proportion of plants homozygous at the *Bm* locus for BtMV resistance,

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was released in 1982. The dominant factor for self-fertility (S^f) (Owen 1942) was retained in C719. In 2000, C37 was crossed to C719. Self-sterile (S^s S^s) C37 was used as the female. In addition, supposed F_1 plants were inoculated with BtMV and only those with resistance retained the self-fertility trait. From five crosses, F_1 plants from crosses 0221-2, 0221-3 and 0221-4 were selfed under paper bags in the greenhouse to produce F_2 seed. From four self-fertilized families (F_2s) for each cross, the F_2 populations 1221-2-2, 1221-3-2 and 1221-4-2 were chosen for marker analysis based on seed quality and quantity. Each of these F_2 populations would have had different C37 and C719 parental plants (germplasm accessions C37 and C719 are available from the USDA, National Plant Germplasm System upon request at http://www.ars-grin.gov/npgs/orders.html). Sugar beet variety ACH 9369 was kindly provided by Dr. John Kern (American Crystal Sugar Co., Moorhead, MN, USA). Sugar beet F_2 populations KWS0082 and KWS9824 from KWS SAAT AG were used for genetic map construction, with KWS9824 segregating for genetic male sterility. Population size was 199 and 181, respectively. Map construction was done with JoinMap 2.0 (Stam and Van Ooijen 1995), using standard parameters as described in the manual. In population KWS9824 mapping was done only with fertile F_2 plants; genetic sterility was scored in fertile F_3 families (i.e. as heterozygous or homozygous fertile) and converted into a marker score.

Linkage of the male sterility (A_1) trait with BtMV resistance: Tests for linkage of morphological traits with *Bm* were carried out at Salinas, CA, USA. Sugar beet plants in the two- to four-true-leaf stage were juice or mechanically inoculated with BtMV as described (Lewellen 1973). Systemic symptoms were scored 7–14 days postinoculation. Incipient symptoms were found best for discriminating differences in reactions. The susceptible class showed stunting, vein clearing, mottling and initial severe mosaic symptoms. The plants that showed no symptoms and greatly delayed symptom expression with a much milder phenotype were assigned to the resistant class. Beet mosaic virus susceptible C37 and resistant line 8500 were used as checks.

Test cross and F_2 populations were produced as described in Fig. 1. The original source of resistance to BtMV, line 8500 (*BmBm*), was crossed to the annual (*BB*) (Abegg 1936), double-haploid sugar beet line C5600 (*bmbm*) (Hammond 1966). Resistant (*Bmbm*) F_1 plants were crossed to the biennial (*bb*) plants from line 7102, which were genetically male sterile (a_1a_1), monogerm (*mm*) (Savitsky 1954), red hypocotyl (*RR*) (Keller 1936) and self-sterile (S^sS^s). This new generation of F_1 plants was tested for reaction to BtMV [20 resistant: 12 susceptible ($\chi^2 = 2.0$, P = 0.16, NS for 1 : 1 ratio)] and only the most resistant F_1 plants (*Bmbm*) were chosen to produce F_2 and test cross generations. Resistant F_1 plants were individually selfed under pollen isolation bags in the greenhouse to produce F_2 populations. These F_2 populations were assigned the numbers 9286, 9287 and 9288 (Tables 1 and 2). Other resistant F_1 plants were test crossed to genetic male-sterile plants from parental line 7102 by pairing under isolation bags and harvesting seed only from 7102. The resulting populations were designated 9276 and 0227 (Tables 1 and 2). The genotype for the 7102 parent was *bmbm* a_1a_1 *mm bb RR* S^sS^s and for the male parent C5600 × 8500 *Bmbm* A_1A_1 *MM BB rr* S^fS^f. Individual plants within the F_2 and test cross families were each scored for reaction to BtMV and male fertility, monogerminity, hypocotyl colour, annual growth habit and self fertility. Appropriate chi-squared tests for goodness of fit were run for 3 : 1 F_2 ratios and 1 : 1 test cross ratios for reaction to BtMV. The six genes known to be segregating in these F_2 and test cross families were examined in all possible pairwise combinations for independent assortment. Where linkage was detected between *Bm* and A_1 loci the percent recombination in the test cross was calculated by dividing the recombination classes by the total number of plants observed. For the F_2 families percent recombination was calculated according to Allard (1956).

BtMV inoculation and phenotypic analysis for marker development: Sugar beet populations 1221-2-2, 1221-3-2 and 1221-4-2 were planted and grown in the greenhouse under natural sunlight supplemented with greenhouse lights to produce a 16-h photoperiod. Plants were grown in Sunshine mix #1 (Sungrow Horticulture, Vancouver, BC, Canada) until the six- to eight-leaf stage, when they were artificially inoculated with BtMV. A strain of BtMV was maintained in the susceptible hybrid ACH 9369. Fresh symptomatic leaves were ground with a mortar and pestle in 0.1 M phosphate buffer (39% 0.1 M NaH_2PO_4 and 61% $NaHPO_4$) containing carborundum and applied to 4-week-old test plants by rubbing the suspension containing the virus onto leaves, mechanically wounding the leaves in the process. Mosaic symptoms were observed 12–13 days after mechanical inoculation and plants were identified as susceptible or resistant based on the presence or absence, respectively, of a typical mottling symptom. Phenotypic results were subjected to a basic chi-squared analysis to test the goodness of fit for a single gene trait.

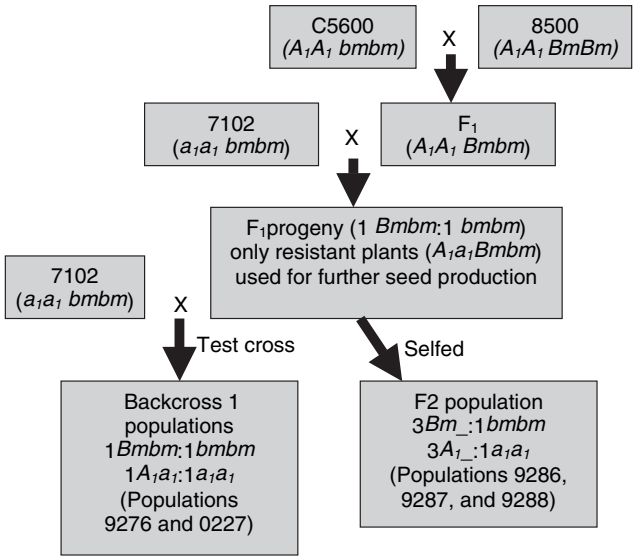

Fig. 1: Flow chart of breeding strategy for population analysis of the *Bm* gene and its association with known morphological traits. F_2 populations 9286, 9287 and 9288 and test cross populations 9276 and 0227 were derived from the scheme

Table 1: Distribution of beet mosaic virus reaction types in sugar beet F_2 and test cross families derived from crosses between resistant and susceptible parents and chi-squared tests for goodness of fit to monohybrid ratios

No. of plants	Resistant[1]	Susceptible[2]	Ratio	χ^2	P value[3]
F_2 populations					
9286	119	39	3 : 1	0.01	0.90–0.95
9287	228	84	3 : 1	0.62	0.25–0.50
9288	548	168	3 : 1	0.90	0.25–0.50
Test cross populations					
9276	158	123	1 : 1	4.36	0.03–0.05
0227	167	152	1 : 1	0.71	0.25–0.50

[1] Probable genotypes for F_2s are *BmBm:2Bmbm* and for test crosses *Bmbm*.
[2] Probable genotype for F_2s and test crosses is *bmbm*.
[3] Probability for calculated chi-squared values.

DNA extraction, bulked segregant analysis and SCAR marker development: Sugar beet DNA was extracted prior to virus inoculation using a CTAB mini-prep extraction method (Doyle and Doyle 1987). Bulked segregant analysis (BSA) (Michelmore et al. 1991) was used in conjunction with random amplified polymorphic DNA (RAPD)

analysis (Williams et al. 1990). The DNA from 21 susceptible plants and 21 resistant plants was adjusted to 10 ng/μl and combined to produce three pools (seven plants per pool) within each disease category. These were subjected to fingerprinting with 10-mer oligonucleotides (Invitrogen Inc., Carlsbad, CA, USA) according to standard protocols (Williams et al. 1990). Amplified products were separated on 1.6% agarose gels using Tris–borate–EDTA electrophoresis buffer and stained with ethidium bromide. Gels were documented digitally with a ChemImager 6000 (AlphaInnotech Inc., San Leandro, CA, USA).

Candidate marker bands were excised from gels and cloned using the TOPO TA cloning kit (Invitrogen Inc.). Sequencing was performed on five clones from each band by Northwoods DNA, Inc. (Solway, MN, USA). Primer DNA for SCAR amplification was synthesized by Invitrogen Inc. Marker DNA was amplified using the specific Rbm (for 'resistance to beet mosaic') primer pairs Rbm06fwd (5′-GCACTGTGCTGTTGCATTCT-3′) and Rbm06rev (5′-GGACTGGAGTGAGGAGGAGAG-3′) or Rbm05fwd (5′-GACTGGAGTCGTAAAAGCACTGT-3′) and Rbm05rev (5′-GGAAGCATTTCATACTCTTTTATGGT-3′) by including 30 ng of each primer in a 30-μl reaction. Each 30-μl reaction contained 40 ng of genomic DNA in 1× *Taq* buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.2 μM of each primer and 1.5 U of *Taq* DNA polymerase (Promega). Cycling conditions included 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. Products were analysed by electrophoresis as described earlier.

For the pyrosequencing assay new primers were designed with the default parameters of PSQ assay design software 1.0.6 (Biotage AB, Uppsala, Sweden). Polymerase chain reaction (PCR) amplifications were done in a 20-μl reaction containing 25 ng of genomic DNA, 0.2 μM of each primer (one primer biotinylated), 200 μM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 1 U of HotStar *Taq* DNA polymerase (Qiagen, Hilden, Germany). The cycling conditions were 94°C for 15 min, 40 cycles of 15 s at 94°C, 30 s at 60°C and 30 s at 72°C, followed by a final extension of 5 min at 72°C. A total of 10 μl of the PCR products was immobilized onto streptavidin-coated Sepharose beads (Amersham Biosciences Europe GmbH, Freiburg, Germany) and single-stranded DNA for pyrosequencing was prepared with the Vacuum Prep Workstation according to the manufacturers protocol (Biotage AB). The single-stranded DNA template was mixed with the sequence primer (5′-TGGAGGAGATCAGACC-3′) and analysed with the Pyrosequencer instrument PSQ HS 96A following the manufacturer's protocol (Biotage AB). To determine the mapping position of the Rbm05 marker, plants of the population KWS0082 were genotyped using the markers described previously. Markers were scored codominantly and integrated into the existing map of SSR markers.

Results

Linkage of morphological traits

As shown in Tables 1–3 and consistent with the results of Lewellen (1973), the reactions to BtMV fit the segregation for a single gene where resistance (*Bm*) is dominant to susceptibility.

The locus for *Bm* was found to be linked to A_1. The percent recombination between *Bm* and A_1 for two sets of F_2 and test cross families ranged from 12.4% to 19.6% (Table 2). Thus, the loci for *Bm* and A_1 would both be on chromosome 1.

Population inoculation with BtMV for marker development

Populations 1221-2-2, 1221-3-2 and 1221-4-2 were evaluated at 12–13 days postinoculation. Two of the three populations (1221-2-2 and 1221-3-2) showed a resistant to susceptible ratio that was not significantly different from the expected 3 : 1 for a single dominant resistant gene trait. Segregation ratios are shown in Table 3.

Table 2: Distribution for reaction to beet mosaic virus and genetic male sterility in the F_2 and test cross individuals of 9102 *bmbm aa* × (C5600 × 8500) *Bmbm AA*[1] and chi-squared tests for goodness of fit to dihybrid ratios

		No. of plants observed			
		Test crosses		F_2 individuals	
Phenotypes	Proposed genotypes	9276	0227	9287	9288
Resist., fertile	*Bm_*, A_1_	126	141	124	166
Resist., ms	*Bm_*, a_1a_1	32	23	16	18
Susc., fertile	*bmbm*, A_1-	23	18	14	10
Susc., ms	*bmbm*, a_1a_1	100	134	35	46
Total no. of plants		281	316	189	240
χ^2 for 1 : 1 : 1 : 1		110**	173**		
χ^2 for 9 : 3 : 3 : 1				72**	114**
% recombination		19.6	13.0	16.9	12.4

ms, male sterile; Resist., resistant; Susc., susceptible.
** Significant at P = 0.01. Loci do not have independent recombination.
[1] F_1 would have segregated *Bmbm:bmbm* but after inoculation tests, only resistant *Bmbm* F_1 plants were used for selfing and backcrossing.

Table 3: Phenotypic disease ratios and association between the beet mosaic virus (BtMV) resistance locus (*Bm*) and marker Rbm05

Population	Total R plants	Total S plants	χ^2 (3 : 1 ratio)	R plants associated with marker (%)	S plants associated with marker (%)	Recombination frequency between *Bm* and Rbm05 (%)
1221-2-2	108	29	1.07	99 (91.7)	2 (6.7)	8.0
1221-3-2	106	24	2.96	104 (98.1)	0	1.5
1221-4-2	107	17	8.43*	101 (94.4)	0	4.8

*Significant at P = 0.05.

RAPD analysis and SCAR marker generation

A total of 180 arbitrary decamer primers were tested using BSA applied to population 1221-2-2. Primer OPB04 gave products that indicated amplification of repulsion and coupling markers associated with the *Bm* gene. Following the cloning and sequencing of the ~0.5-kbp RAPD product associated with *Bm*, longer specific primers (pair Rbm06fwd and Rbm06rev and pair Rbm05fwd and Rbm05rev) were designed and tested for the ability to detect sequence polymorphism associated with this trait. As shown in Fig. 2 (top), both the coupling (~0.5 kbp) and repulsion (1.2 kbp) associated products were amplified by primer pair Rbm06. These were subsequently cloned, sequenced and analysed for sequence similarity. This analysis revealed that the marker sequence associated with the resistance allele lacks 748 nucleotides that are nested within the sequence of the repulsion associated marker (Fig. 2).

Although primer pair Rbm06 amplified products with both a coupling and repulsion association with resistance, PCR amplification was not as reliable as that for primer pair Rbm05 (Fig. 2, bottom), which detected only the resistance allele. For this reason, only primer pair Rbm05 was used to screen individuals from the three populations tested in order to assess linkage values between the resistance trait and the marker. The results are shown in Table 3. Only within population 1224-2-2 was the marker associated with resistance detected in two susceptible plants; the marker was not detected in susceptible plants of populations 1224-3-2 or 1224-4-2. In resistant plants of all three

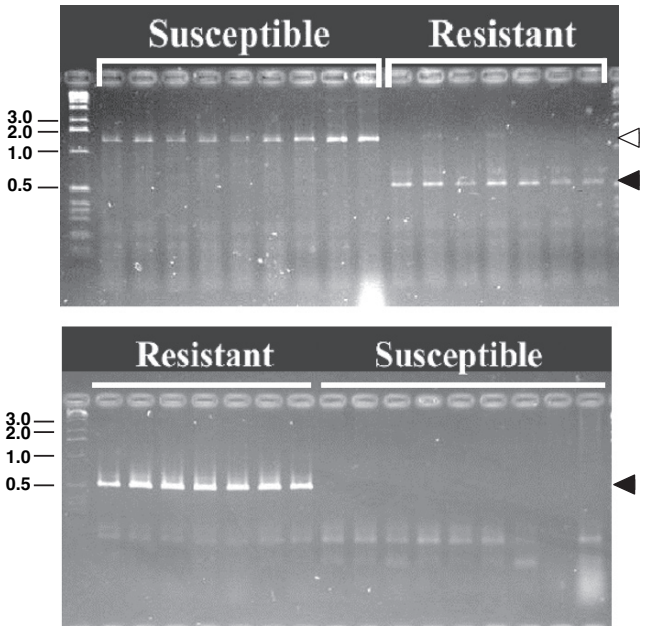

Fig. 2: Amplification products from DNA of individual plants resistant and susceptible to beet mosaic virus using the Rbm06 primer set (top) and the Rbm05 primer set (bottom). Products were separated by electrophoresis on a 1% agarose gel. The filled arrow denotes the product associated in coupling with resistance and the open arrow denotes the product associated in repulsion with resistance. DNA fragment size in kilobasepairs is indicated on the left side of the panels

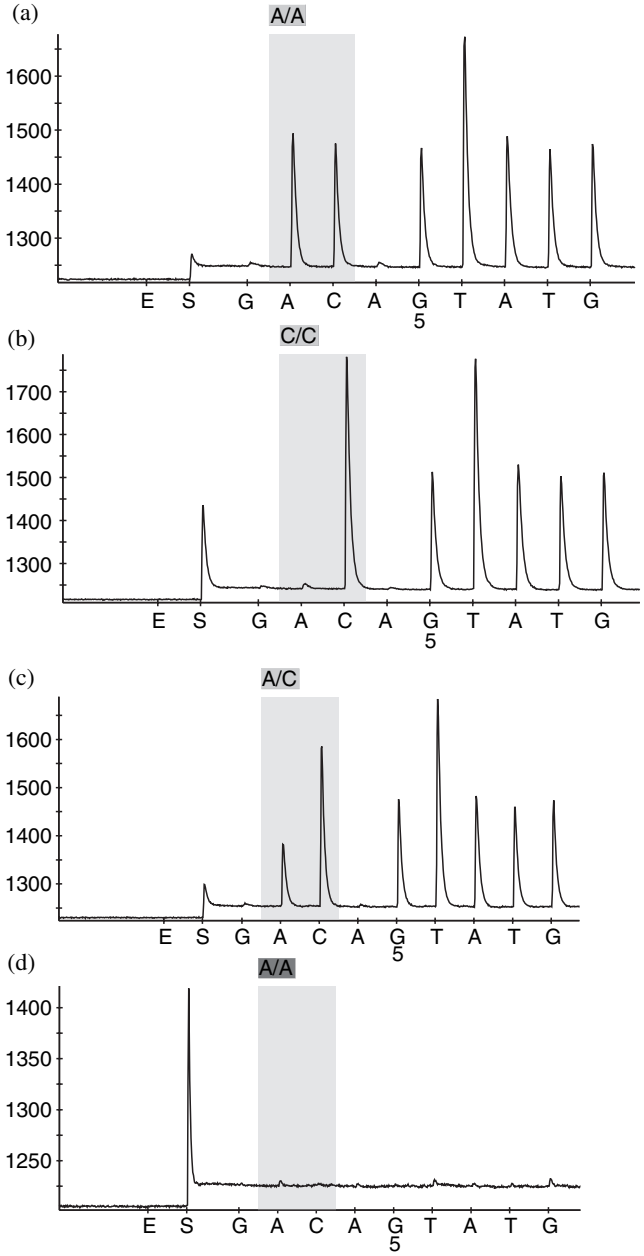

Fig. 3: Pyrogram pattern of F2 mapping plants analysed with the pyrosequencing marker for Rbm05: (a) homozygous A, (b) homozygous C, (c) heterozygous and (d) water control

populations the marker was detected in 91.7%, 98.1% and 94.4% of populations 1224-2-2, 1224-3-2 and 1224-4-2, respectively. The nucleotide sequence of the Rbm05 marker is available under accession #DQ022571 through the GenBank sequence database (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

Even though the Rbm05 amplicon was generated from the DNA of both parents of mapping population KWS0082, a single-nucleotide polymorphism (SNP) was found at position 188 (GenBank accession #DQ022571) that distinguished the parents and progeny of this population. Scoring of this SNP could be done in a codominant manner because every genotype of the population showed a specific pyrogram pattern based on a C/A nucleotide polymorphism (Fig. 3). The same C/A polymorphism was also found to occur in the 1221-2 series, which segregated for the *Bm* gene in this study (data not shown). By genotyping 200 F_2 plants of the mapping population KWS0082, using the pyrosequencing assay for Rbm05, the marker could be assigned to beet chromosome 1 (Fig. 4). Since the Rbm5 marker was not polymorphic in the KWS9824 population, linkage to the genetic male sterility locus could be estimated from the relative distance to two anchor markers (kws1091 and kws3217) common to populations KWS9824 and KWS0082. The two anchor markers were polymorphic in population KWS9824, confirming that genetic male sterility (A_1) also maps on chromosome 1. The distance between Rbm05 and A_1 can be estimated at 20–25 cM. Recombination values between Rbm05/*Bm* and the A_1 allele were found to be consistent when comparing results obtained using phenotypic data (Table 2) and those using marker data (Fig. 4).

Discussion

Inheritance studies conducted by Lewellen (1973) concluded that one dominant or incompletely dominant gene, *Bm*, conditioned resistance to all isolates of BtMV tested. This study also showed that concentrations of the virus are reduced by the *Bm* gene and that the heterozygous F_1 had a slightly elevated virus concentration as compared to the homozygous *BmBm* parent. In our study based on visual symptoms of the virus no intermediate class was observed. It is possible that an intermediate virus concentration was present but that visual symptoms did not show an intermediate phenotype. The intermediate reaction was more notable in the original inheritance studies as infection approached the chronic phase. Resistance to the potyviruses can be genetically dominant, recessive or semidominant (Lewellen 1973, Fraser 1990), with recessive resistance conditioned in some cases by null mutations in eukaryotic translation initiation factor (iso)4E

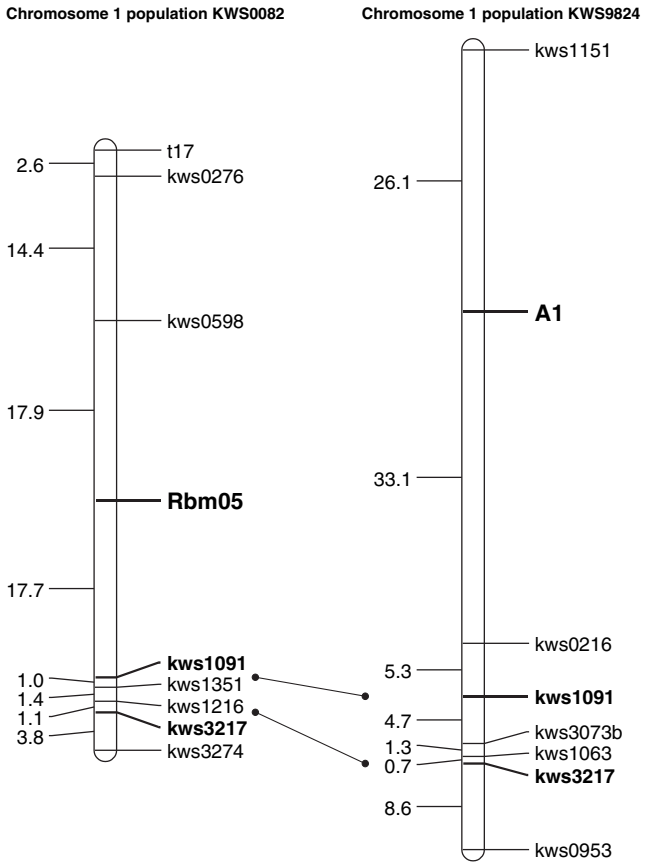

Fig. 4: Linkage maps of *Beta vulgaris* chromosome 1 derived from populations KWS0082 and KWS9824 used to localize both Rbm05 and A1 locus for genetic male sterility, respectively. Distances between markers are given in centimorgans

(Duprat et al. 2002). Dominant/semidominant resistance provided by the *Bm* gene in beet is similar in phenotype to the resistance to turnip mosaic potyvirus in oilseed rape (Walsh 1989, Hughes et al. 2003). In both cases, the mechanism of resistance remains unknown.

Segregation ratios in all populations were slightly skewed towards resistance, with population 1221-4-2 being significantly different from a 3 : 1 ratio. This result can be explained in three different ways. Because of the skewed segregation towards resistance it is possible that more than one gene is conferring the resistance to BtMV. This is probably not the case because the ratios are also not significant for two dominant resistant genes, although the presence of genes modifying a major gene cannot be ruled out. Secondly, plants that were disease-free and rated as resistant could represent progeny that were actually susceptible but escaped disease development for unknown reasons. Usually, local lesions were produced on all inoculated plants and were used to rule out the occurrence of escapes. Lastly, the skewed segregation could be due to segregation distortion in the chromosomal region where *Bm* is located. Segregation distortion occurs when independent loci do not segregate in a Mendelian fashion. Segregation distortion near *Bm* is likely to occur because the phenomenon is commonly reported in sugar beet (Oleo et al. 1993, Pillen et al. 1993) and other plant populations (Faris et al. 1998, Lu et al. 2002) and because it is supported by molecular marker data where the number of progeny containing markers linked to the *Bm* gene are skewed similarly as the phenotypic data. The result is similar to the characterization of a Let5 locus for distorted segregation cited in Schondelmaier and Jung (1997).

The A_1 trait was assigned to linkage group III by Theurer (1968b). The A_1 gene was the only morphological marker found on this linkage group. To our knowledge, *Bm* is only the second morphological trait known for this linkage group. Marker Rbm05 potentially will be useful in marker-assisted selection programmes aimed at introgressing both of these genes in sugar beet parents and hybrids. Because of the difficulty of manual emasculation of sugar beet flowers, genetic male sterility is very important and is widely used by sugar beet breeders. A very closely linked marker to A_1 may also be very useful in some breeding and seed production schemes to produce hybrid cultivars of sugar beet. Marker-assisted selection offers a faster, less labour-intensive method of introgressing this resistance gene into sugar beet lines. Additionally, it is anticipated that Rbm05 will find use in efforts to characterize the sugar beet genome at or around the *Bm* locus. This marker can also be used in the future work of map-based cloning and characterizing the mechanism of resistance of the *Bm* gene and male sterility at the A_1 locus.

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